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PRINCIPAL INVESTIGATOR: Mineo Iwata, PhD

CONTRACTING ORGANIZATION: FRED HUTCHINSON CANCER RESEARCH
CENTER
Seattle ,WA 98109-4433

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14. ABSTRACT <p>Hematopoietic stem cells (HSC) and their progeny reside in specialized niches of the microenvironment (ME) in the bone marrow. The ME niches control HSC self-renewal, differentiation and maturation. The ME niche cells were derived from non-hematopoietic cells, including fibroblasts, osteoblastic and endothelial cells. Macrophages, which are hematopoietic in origin, are also a critical component of the ME, and can influence the function of the ME niche cells. I hypothesize that the macrophages can acquire defects that may compromise ME function and lead to bone marrow failure. To test this hypothesis, I proposed to develop a new in vivo model that allows the inducible depletion of the macrophages in dogs, followed by the documentation of marrow failure, and subsequent therapeutic interventions. At this period of the grant support, I achieved 4 goals: (1) Optimize culture conditions for generating dog macrophages, (2) Optimize transduction efficiency of a macrophage specific CD163 promoter construct in dog CD34+ HSC, and test its macrophage-specific expression, (3) Establish a Luciferase-reporter assay to test the macrophage-specific promoter activity, and (4) Generate multiple lentiviral vectors containing the dog/human CD163promoter, iCasp9 and p140MGMT constructs.</p> <p>I am currently in the final step to generate a lentiviral construct that incorporates both a constitutive selectable marker (p140MGMT) that will increase the proportion of the transduced cells in marrow, and an inducible macrophage-specific promoter that will drive the expression of a suicide gene (iCasp9). In the original grant application, I proposed to use the CD68 promoter sequence to drive the macrophage specific expression of the inducible iCasp9 suicide gene. The CD68 promoter, however, is intronic and clones into a reverse orientation in the lentiviral vector to avoid splicing during retroviral packaging. While I attempted to clone this promoter sequence in the reverse orientation using different conditions, the cloning was not successful, likely due to the toxicity of the DNA in the bacteria. I have shifted my focus to work with another macrophage specific promoter for CD163/hemoglobin scavenger receptor. The CD163 gene promoter has been reported, and is macrophage specific. It is not intronic, but locates 1.4 kb region upstream of the ATG translation start site. The CD163 promoter sequences in human and dog were cloned, tested for the macrophage-specific expression by Luciferase assay. I am currently constructing multiple lentiviral vectors containing the CD163promoter-iCasp9 and p140MGMT constructs. Once this goal is achieved, I will make the lentiviruses on a large scale, and perform an autologous transplant in two dogs using dog CD34+ cells transduced with the vector, then induce expression of the suicide gene and follow the consequences to marrow function.</p>					
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INTRODUCTION

Hematopoietic stem cells (HSC) and their progeny reside in specialized niches of the microenvironment (ME) in bone marrow. The ME niches control HSC self-renewal, differentiation and maturation. The cells of the ME niche are derived from non-hematopoietic cells, including fibroblasts, osteoblastic and endothelial cells. Macrophages, which are hematopoietic in origin, are also a critical component of the ME, and can influence the function of the ME niche cells. I hypothesize that the macrophages can acquire defects that may compromise ME function and lead to bone marrow failure. To test this hypothesis, I proposed to develop a new *in vivo* model that allows the inducible depletion of the macrophages in dogs, document the marrow failure, and conduct subsequent therapeutic interventions.

BODY:

Goal 1: Optimize the culture conditions for generating dog macrophages

Canine peripheral blood mononuclear cells (PBMCs) were cultured in two different media, Medium-I and Medium-II (Table 1). Medium-I was formulated and modified based on the media for human hematopoietic cells, and Medium-II was formulated based on a previous mixture used for dog hematopoietic cells (Abrams et al, 2010). Figure 1 shows that Medium-I is better for expanding dog PBMCs than Medium-II. Additionally, Figure 2 shows that the progeny of CD34+ hematopoietic stem/progenitor cells increased 4-5 fold in 7 days when cultured in the Medium-I. These data show that the Medium-I is a good choice for this study.

Next, I tested if human macrophage colony-stimulating factor (hMCSF) induces the differentiation from dog hematopoietic progenitors to macrophages. When dog PBMCs were cultured in hMCSF, 4 fold more dog macrophages than the controls were generated (magenta bars in Figure 1). When dog CD34+ (dCD34+) cells were cultured in hMCSF, more than 90% of the adherent cell population became CD163+/CD14+ macrophages (Figure 3). The macrophage number increased 10 fold in the presence of hMCSF. These data show that human MCSF is useful for generating dog macrophages *in vitro*.

Goal 2: Optimize transduction efficiency of a macrophage specific promoter construct in dog CD34+ HSC/HPC and test its macrophage-specific expression by Luciferase assay

In my initial application, I proposed to use the human CD68 promoter to drive the expression of the inducible suicide gene known as Caspase 9 (iCasp9). The GFP reporter construct under the control of CD68 promoter was originally obtained from Dr. Elaine Raines of the University of Washington (Gough and Raines, 2003). A lentiviral construct of the vector was created, and a high titer lentiviral preparation was made. Dog CD34+ cells were then transduced and cultured with hMCSF for 6 days. Figure 4 shows that the transduced cells expressed GFP and CD14+ monocyte/macrophage specific marker, assuring macrophage specific expression of the reporter construct.

However, the CD68 promoter turns out to be problematic. As I described in the Abstract, the CD68 promoter is intronic and clones into a reverse orientation in the lentiviral vector to avoid splicing during retroviral packaging. While I attempted to clone this promoter sequence in the reverse orientation using different conditions, the cloning was not successful, likely due to the toxicity of the DNA in the bacteria. I shifted my focus to work with another macrophage specific promoter for a gene called CD163/hemoglobin scavenger receptor in order to avoid the cloning issues I have had with the CD68 promoter. The CD163 gene promoter has been previously studied (Gronlund et al, 2000), and is macrophage specific. It is not intronic, and is located in the 1.4 kb region upstream of the ATG translation start site. Preparation of the human and dog CD163 promoter constructs is described in the next section of Goal 3.

I conducted optimizations for the macrophage specific constructs containing the CD163 promoter. Figure 5 shows that the U937 myelocytic leukemia line can differentiate into CD163+ macrophages by using 12-O-tetradecanoylphorbol-13-acetate (TPA). U937 cells can be transfected by using the nucleofector (Amaxa, Lonza, Cologne, Germany), and CD163 reporter activities were detected by Luciferase-based assay (GeneCopoeia, Rockville, MD) (Figure 6). These data show that the CD163 promoter constructs are functional in CD163+ cells.

Goal 3: Prepare DNA constructs containing the CD163promoter, iCasp9 and p140MGMT

I obtained the plasmid containing iCasp9 from Drs. Elizabeth Budde and Caroline Berger of the Fred Hutchinson Cancer Research Center (FHCRC) (Figure 7), and the plasmid containing p140MGMT from Dr. Hans-Peter Kiem of the FHCRC (Beard et al, 2009). The vector containing human CD163 promoter construct was purchased from GeneCopoeia. Dog and human CD163 promoters were cloned as described below.

Figure 8 shows diagrams of the lentiviral vectors. They contain the CD163promoter-iCasp9 and p140MGMT constructs. Dog and human CD163 promoters have 70% identity (Figure 9), and both promoters were

cloned into the TOPO vector (Figures 10 and 11). The iCasp9 fragment was amplified from the plasmid given by Drs. Budde and Berger as shown in Figure 11. However, as the annealing of the iCasp9 to CD163 promoter and p140MGMT vectors was not successful, I modified and optimized the iCasp9 coding region by gene sequencing (Genscript, Piscataway, NJ). The optimized synthetic fragment of iCasp9 was annealed and cloned into the lentiviral vector of p140MGMT (Figure 12). Human and dog CD163 promoter constructs are being inserted into the iCasp9-p140MGMT lentiviral vector.

Current:

I am currently in the final process of making multiple lentiviral vectors containing the final CD163promter-iCasp9 and p140MGMT constructs. These vectors will be sent to the virus production core of the Core Center for Excellence of Hematology (CCEH) at the FHCRC to make the lentiviruses on a large scale for an autologous transplant in two dogs. Dr. Brian Beard at the virus production core told me that it would take 2-3 weeks to make the viruses. Once the viruses are made, I will perform an autologous transplant in dogs using dog CD34+ cells transduced with the viruses, then induce expression of the iCasp9 suicide gene and follow the consequences to marrow function.

KEY RESEARCH ACCOMPLISHMENTS:

At this period of the grant support, I achieved 4 goals, as follows:

- Optimized culture conditions for generating dog macrophages
- Optimized transduction efficiency of a macrophage specific promoter construct in dog CD34+ HSC/HPC, and tested its macrophage-specific expression
- Established Luciferase-reporter assay to test the macrophage-specific promoter activity
- Constructed multiple lentiviral vectors containing iCasp9 and p140MGMT constructs, and plasmids containing the dog/human CD163promoter.

REPORTABLE OUTCOMES:

None at this grant period.

CONCLUSION:

At this period of the grant support, I have optimized the culture conditions for generating dog macrophages, and transduction of dog and human hematopoietic cells to test macrophage specific promoter activity. I am in the final process of constructing the lentiviral vectors that contain the CD163 promoter-iCASP9 and p140MGMT constructs. The constructs will be sent to the virus production core of CCEH at the FHCRC. Once the lentiviruses are made, I will perform an autologous transplant in dogs using dog CD34+ cells transduced with the viruses, chemoselect to increase gene marked cell frequency, then induce the macrophage specific expression of the suicide gene and follow the consequences to marrow function.

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- Abrams et al (2010) A novel monoclonal antibody specific for canine CD25 (P4A10): Selection and evaluation of canine Tregs. *Vet Immunol Immunopathology* 135, 257-265
- Beard et al (2009) Long-term polyclonal and multilineage engraftment of methylguanine methyltransferase P140K gene-modified dog hematopoietic cells in primary and secondary recipients. *Blood* 113, 5094-5103
- Gough and Raines (2003) Gene therapy of apolipoprotein E-deficient mice using a novel macrophage-specific retroviral vector. *Blood* 101, 485-491
- Gronlund et al (2000) Cloning of a novel scavenger receptor cysteine-rich type I transmembrane molecule (M160) expressed by human macrophages. *J Immunology* 165, 6406-6415

APPENDICES:

None

SUPPORTING DATA:

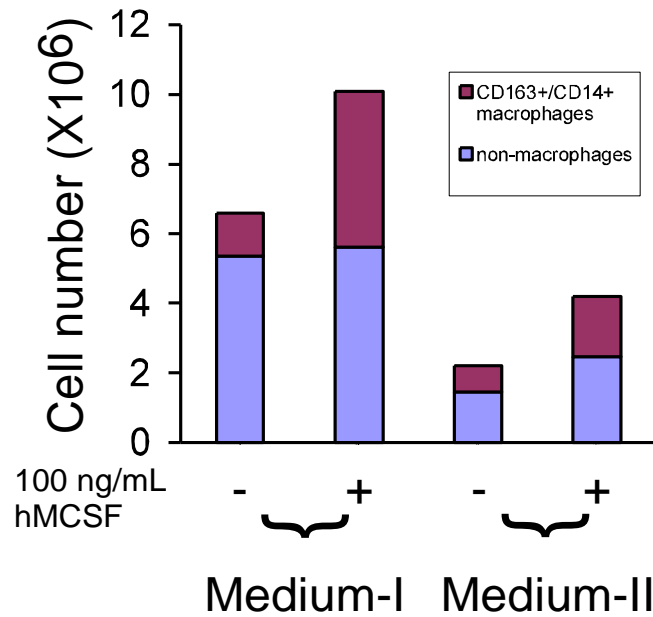


Figure 1

Human macrophage colony-stimulating factor (hMCSF) increases dog macrophage numbers *in vitro*. Dog bone marrow mononuclear cells (5 millions) were cultured for 7 days in the media described above, and the cell numbers were counted. The cells were analyzed using flow cytometry to identify macrophages (CD163+/CD14+ cells). The cells in Medium-I showed 2-4 fold greater expansion than those of Medium-II. From these data, hMCSF in Medium-I was chosen to be used in the following experiments.

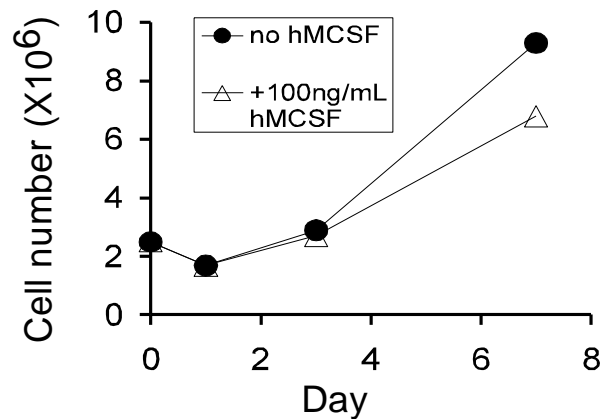


Figure 2

Expansion cultures of dog CD34+ HSC/progenitor cells (dCD34+ cells). Dog CD34+ cells (2.5 millions) were cultured in Medium-I +/- 100ng/mL hMCSF, and the cell numbers were counted at each time point described on the X-axis. After 7 days of culture, a 3- to 5-fold expansion of the cells was achieved.

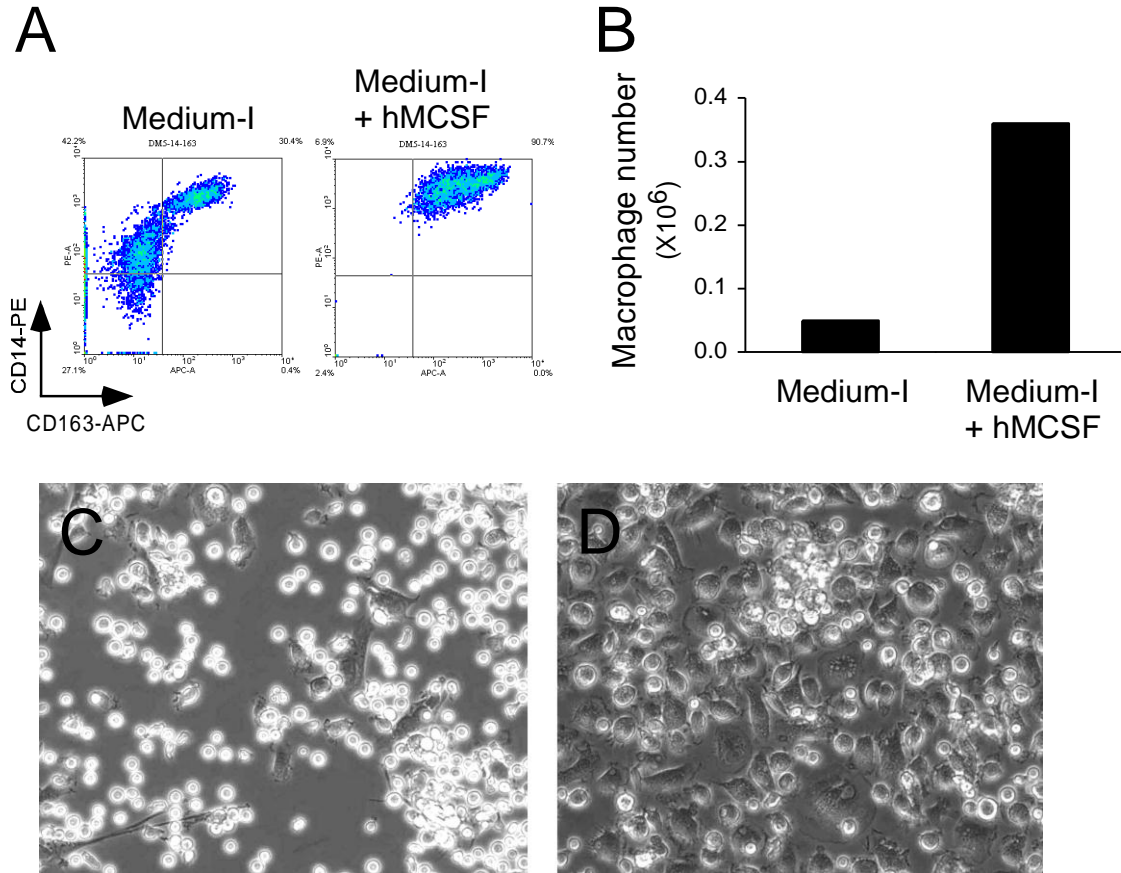


Figure 3

***In vitro* generation of canine macrophages from dCD34⁺ cells.** dCD34⁺ cells were cultured in Medium-I in the presence and absence of hMCSF (100 ng/mL) for 7 days. The cells were harvested and analyzed for CD14 and CD163 expression by using flow cytometry (panel A). Macrophages are defined as CD163 bright/CD14 bright cells in the upper right quadrant. In the presence of hMCSF, all of the cells differentiated to macrophages. Panel B shows the number of macrophages at Day 7. Panels C and D show phase-contrasted images of the cells cultured in the absence and presence of hMCSF, respectively. The images were captured with an inverted microscope using the X20 objective.

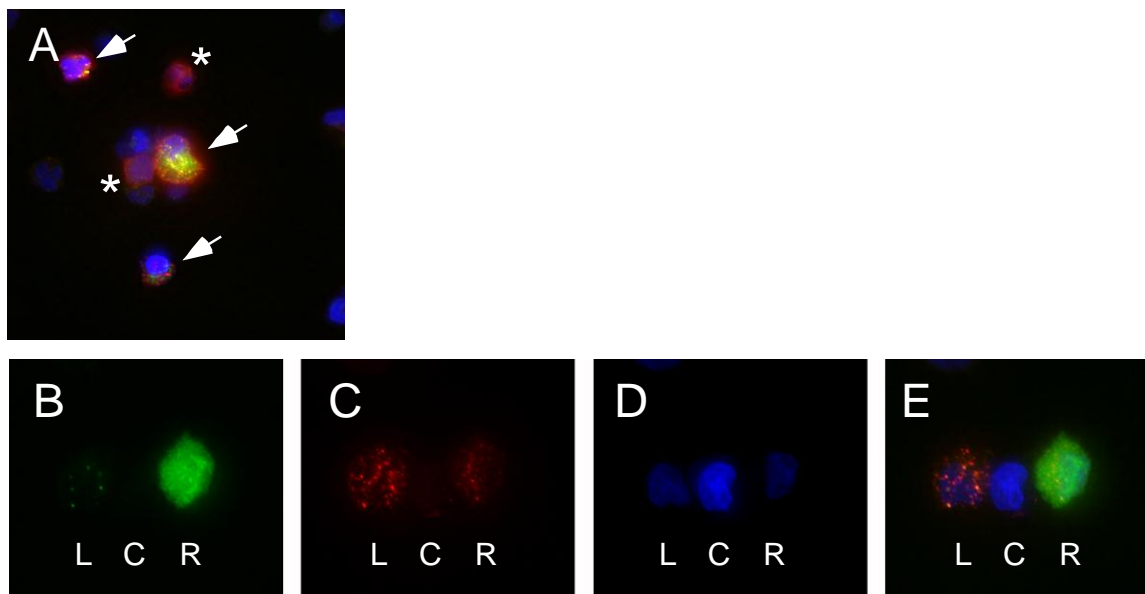


Figure 4

Macrophage specific expression of the CD68 promoter-GFP construct. dCD34+ cells were infected with the lentivirus containing GFP driven by the CD68 promoter. The cells were cultured in Medium-I in the presence of 100 ng/mL of hMCSF for 6 days, and stained for CD14 (red). **Panel A:** Arrows indicate the infected cells (GFP+) co-expressing CD14. Asterisks indicate uninfected CD14+ monocytes/macrophages. (X40 objective) **Panels B-E:** Images taken with a high magnification (X100 objective) of the same specimen as in Panel A are shown. The cell on the left (L) is an uninfected CD14+ monocyte/macrophage. The cell in the center (C) is uninfected and non-myeloid. The cell on the right (R) is an infected monocyte/macrophage (GFP+/CD14+). Panel B shows GFP, Panel C CD14 (red), Panel D nucleus (blue), and Panel E is combined.

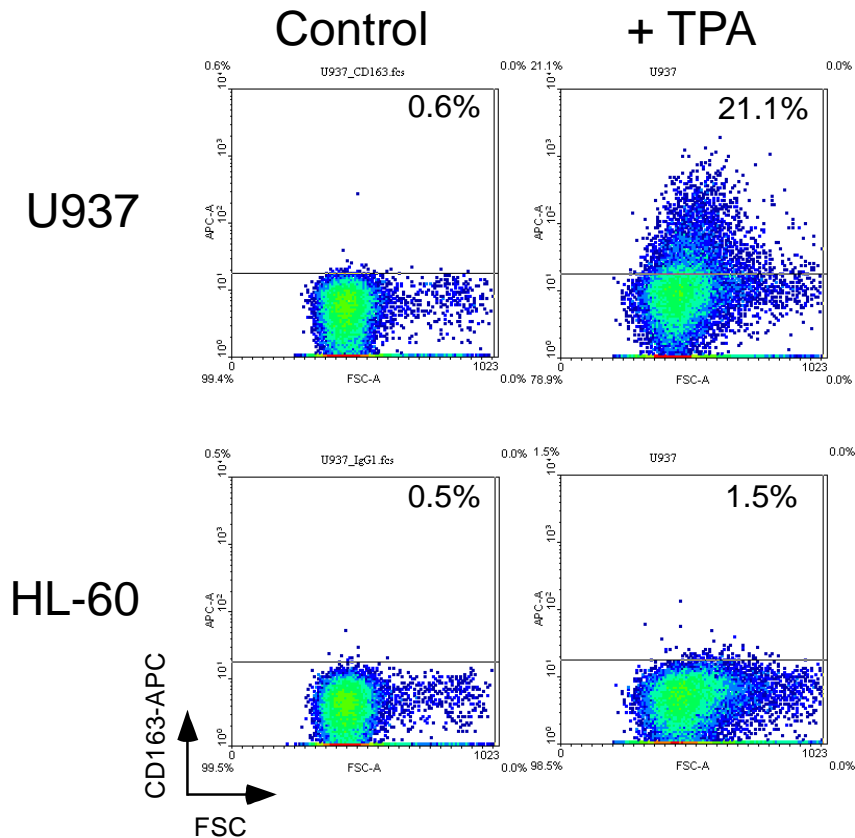


Figure 5

The phorbol ester, 12-o-tetradecanoylphorbol-13-acetate (TPA), induces differentiation of U937 cells (human myelocytic cell line) to CD163+ macrophage-like cells. U937 and HL-60 (human promyelocytic leukemia line) cells were cultured in the absence and presence of 100ng/mL TPA for 3 days. Surface expression of CD163 was determined by using flow cytometry. More than 20% of U937 cells became CD163+ after TPA stimulation. HL-60 cells, in contrast, did not change CD163 expression after TPA stimulation.

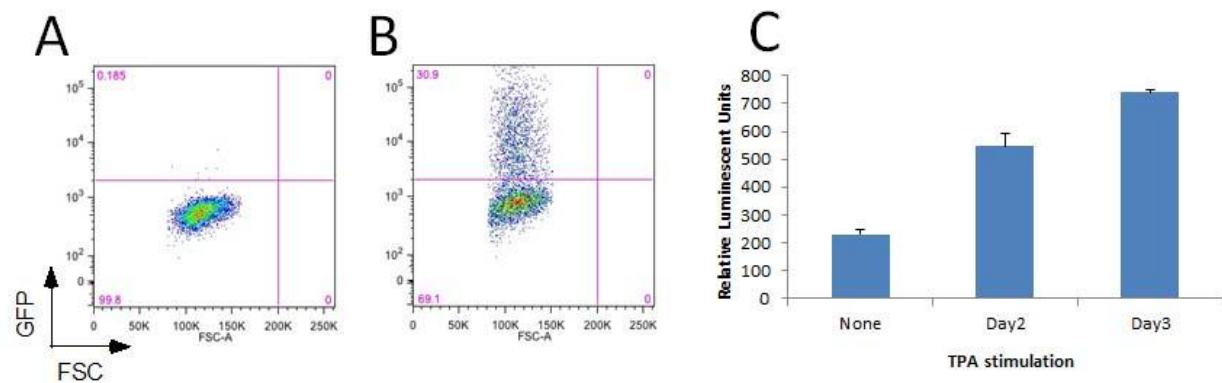


Figure 6

Transduction of U937 cells with pMac-GFP and HPRMCD163 plasmids. U937 cells were transfected with pMac-GFP and HPRM-CD163 plasmids by nucleofection (Nucleofector II, using the program W-01) in the nucleofection solution with supplement in the Kit C (Amaxa). After 6 hours of culture, GFP expression of the untransduced (panel A) and transduced (panel B) cells was measured by using flow cytometry. More than 30% of the cells were transduced and express GFP. Panel C shows Luciferase reporter assay. The cells were stimulated with 50 ng/mL of TPA and harvested at the time point indicated in X axis. CD163 promoter activities were detected by Luciferase assay (Secreta-Pair Dual Luminescence Assay Kit, GeneCopia).

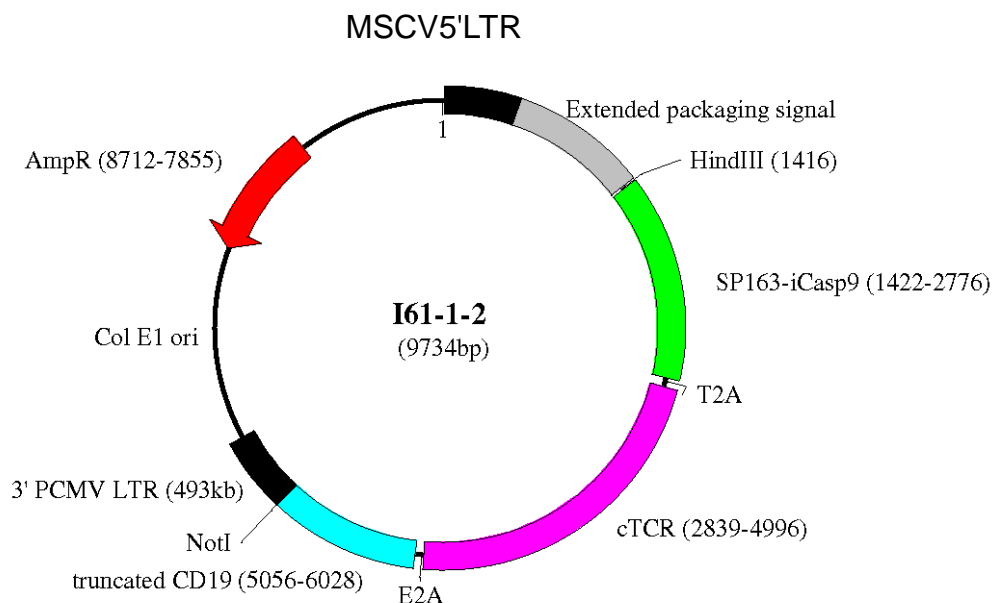


Figure 7.

Schematic diagram of the vector containing iCasp9 suicide gene. Gifted from Dr. Elizabeth Budde at the FHCRC.

Hs163	452	ATTTGAGCCCATCACTAACACCTGACAATATGTATGCCATGATGAACCTATTAGTAGTTC	511
Cf163	508	----GAATTTCCATGTTTTTACATGCCTGTATTGTTGGTATATGtcttttcttatactt	563
Hs163	512	TTTTGAGCTTATATGTTTTTATATACAAATATTCATTTGTATGTG-CTATTTTATGCCT	570
Cf163	564	taaatatttttatcttttttc--tttGGGGGCAAAGTC---CTCATTATTAGGACACAA	618
Hs163	571	TAAACTCCTTTATCATTTGTCCCTTTGG---CAATGTTTTTCTCATCTTTTAGGACACAA	627
Cf163	619	TTTAAGCACCTTCTCACAGAAAAGAGTTTTCTGACTATTCATTTCTGTCTCCAGTTGAT	678
Hs163	628	TTTAAGCCTGTCTCAGAGAAAATAGTTTTCTGACTGTTCATTCCTTTCTCTCAATAGAT	687
Cf163	679	ATGACCTGTTTTTCTTTATTTTCACTCTGTGCTTGAATCGCTCTGGATTATTGCTCA	738
Hs163	688	AGGACTACTTTGTCCATTATTTTAACTGCACCTTAGCTTTATGTTTCATTGTTGTTAT	747
Cf163	739	CATATGTGTTTCTTTCACT-GAATTTGAGTTATTCAGAGCTAAAATTACGCTTTATAAAT	797
Hs163	748	CATATGTGTTTCTTCTACTTGAATCTGANTTATATGGAGCTAAAATCATGTGTTGCTAAT	807
Cf163	798	TTTTATTGCACCATTTGTAGAATCTGTAATGGTCATGGCTAATTTATTGTTACTCTTCA	857
Hs163	808	TTTTGTTTACCATTGTGAATATCAGTAATAGTCATGGCTAATCTCTTGGTGTACTTCA	867
Cf163	858	TCTCATTAGAAAAGAGAAAGCAAATGCCTTCTGTAGATGGCTACACAAAATTATTCATT	917
Hs163	868	TCCCATTAGAAAAGAAATGACAAATGCTGTGTCTCAACAACCTTACACAAAATTACTCATT	927
Cf163	918	CAGCTCATTTGATTATGGTAATAGTATTTAAAGTGATGAACTGATAAAACATTATTTAA	977
Hs163	928	ANACACATTTGATTATGGAATAAAATTAAAG---TGCATATGATAAAATGTTATTTAA	984
Cf163	978	TTATGTTTTGCTTATTTCACTTTAGTTTTTGTACATAA---CTGCACAGTGA---TAGTC	1030
Hs163	985	TTATGTTTTGCCTGTTTTGCTTTAGTTTTTACATAATTTTCTACA-TGACAATTAGTA	1043
Cf163	1031	A-TTTTTATTCTAATATATTACTCCAAAACAAAGTATGGAAATCT-AAATATTCATTTT	1088
Hs163	1044	ATTTTTTGTGTCTTATATATTTGTCCAAAATGAAGTTCAAAAATGTAATAATTTAATTC	1103
Cf163	1089	AATAGCAACGGAACATGCATTAGTATTTCCCTTAATTTTGTAAATCTGT---AGTGT	1144
Hs163	1104	AGCAACAGCAGCATATGAGTTAGTATTTCTCT-TAATTTTTCGAAATCTGTGGGAAGTGT	1162
Cf163	1145	TTCTCAATTTCTTTTGGTTGTTTCATGTCCCAAATGAAGAAAACATGAGTATGAAAGGG	1204
Hs163	1163	TTCCCAATTTCTTTGTTGTTTCATGTGTATATGAAGAAAACATGAGTATGAAATGG	1222
Cf163	1205	AACCTCAG-TTTGTGAATGACTTCCCTTTTTCGTTGATTGACTCCACCTCCTTTATGTA	1263
Hs163	1223	AACCTCAGCTCTTTCAATGACTTCCCTTTTGTAG---TTGACTCCGCCTCC-ATATGTA	1277
Cf163	1264	GCCTTCTGGGGTTTCTGTTGTGTTTGTCTTGTCTTGTGGAAATGAGATGATT	1323
Hs163	1278	GCC-----TTTCATT-----TCATGAAAGTGAAGTGAAT	1308
Cf163	1324	TTTAGAATTCCTTAGTGGTCCTCTTAGCAGAACACTTCTAAGGAATAATACAAGAAGATT	1383
Hs163	1309	TTTAGAATTCCTTAGTGTGTTTCTTTAGAAGAACATTTCTAGGGAATAATACAAGAAGATT	1368
Cf163	1384	TAGAAATCATTAATACTCTGGACTGGACAACTCAGCTCGAGATCT	1429
Hs163	1369	TAGGAATCATTTGAAGTTAT-----AAATCTTTGGCTCGAGATCT	1407

Figure 9.
Comparison of Dog CD163 promoter to Human CD163 promoter. 1.4kb upstream of the non-coding promoter region before ATG translation start site was aligned between dog (Cf) and human (Hs). The CD163 promoter from

human is shown to be specific for macrophages (Gronlund et al 2000). The dog CD163 promoter has 70% identity to the human promoter.

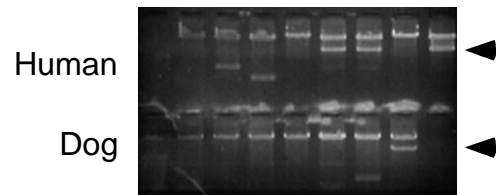


Figure 10

Cloning of human and dog CD163 promoters by PCR from genomic DNA. Human and dog genomic DNAs were PCR-amplified using the primers described in Table 2. The PCR conditions were 95°C/5 min, followed by 35 cycles of 98°C/10 sec, 58°C-64°C step gradient/45 sec and 72°C/1 min in GC buffer (BioLine) with Velocity DNA polymerase (BioLine). PCR products were cloned into the TOPO cloning vector (Invitrogen). Restriction digests of 8 clones for both species are shown. The arrowheads indicate the position of the promoters.

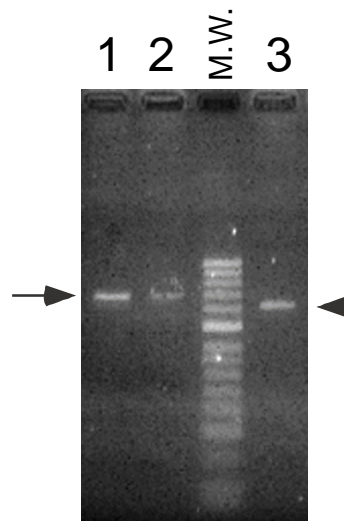


Figure 11

PCR amplification of CD163 promoters and iCasp9 construct. Lane 1: dog CD163 promoter. Lane 2: human CD163 promoter. Lane 3: iCasp9 construct. The arrow indicates the position of the CD163 promoters (14 kb), and the arrowhead indicates the position of the iCasp9 (12 kb). M.W. stands for molecular weight markers (BioLine Hyperladder II, 50 bp to 2000 bp). MiFi taq polymerase (BioLine) was used. PCR conditions were 95°C/5 min, and then 35 cycles of 98°C/10 sec, 62°C/45 sec and 72°C/1 min.



Figure 12

Restriction enzyme digestion of the plasmids containing the optimized synthetic iCasp gene. The optimized iCasp construct was designed and commercially synthesized (Genscript). The construct was cloned into p140MGMT lentiviral vector after cutting with EcoRI. Thirty clones were picked up and digested with BamHI and Nhe I to identify clones containing the insert with the correct orientation. Three such clones are indicated by asterisks. The clones were confirmed by sequencing. Hyper Ladder I and II (BioLine) were used to determine the molecular weights.

Table 1. Culture media used for dog hematopoietic cells.

Medium-I		Medium-II	
Iscove's medium	500 mL	Iscove's medium	450 mL
FCS	50 mL	Waymouth's medium	450 mL
Penicillin/Streptomycin solution	5 mL	Dog serum	100 mL
Glutamine, 200 mM	5 mL	Penicillin/Streptomycin solution	10 mL
Sodium pyruvate, 100 mM	5 mL	Glutamine, 200 mM	10 mL
human IL-3	200 ng/mL	Sodium pyruvate, 100 mM	10 mL
human IL-6	100 ng/mL	Non-essential amino acids	10 mL
human SCF	100 ng/mL		
human Flt3-L	100 ng/mL		
dog G-CSF	100 ng/mL		
human TPO	100 ng/mL		

Table 2. Primers used in this study

Name		Size (bp)	Sequence*
Human CD163 promoter	F	36	CCA GGT ACC GCT AGC <u>AGC ACA CCA GCA TTG CAC ATG</u>
	R	40	CCA AGA TCT <u>CGA GCC AAA GAT TTA TAA CTT CAA TGA TTC C</u>
Dog CD163 promoter	F	40	CCA GGT ACC GCT AGC <u>CAA GTG CTA TCC GCA AGG GCT GGT G</u>
	R	34	CCA AGA TCT <u>CGA GCT GAG TTT GTC CAG TCC AGA G</u>
iCASP	F	29	ATGGGAGTGCAGGTGGAACCATCTCCCC
	R	35	GCT CGA GCG GCC GCT CTT ATG ATG TTT TAA AG

* Underbar shows the unmodified sequence from the promoter.